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## Arbuscular mycorrhizal symbiosis increases the content of volatile terpenes and plant performance in *Satureja macrostema* (Benth.) Briq.

[La simbiosis micorrícica arbuscular aumenta el contenido de terpenos volátiles y el rendimiento vegetal en *Satureja macrostema* (Benth.) Briq.]

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**Abstract:** We studied the effect of *Rhizophagus irregularis* on plant performance and volatile terpenes content of the Mexican native medicinal plant *Satureja macrostema* (Benth.) Briq. (Lamiaceae) in greenhouse conditions. The growth parameters considered in this research and the composition of volatile components were quantified monthly in mycorrhizal and non-mycorrhizal plants. The essential oil was collected from aerial parts and analyzed by gas chromatography-mass spectrometry. Colonization by *R. irregularis* significantly increased biomass, shoot and root length, and the amount of volatile terpenes. The more concentrated volatile terpenes were limonene,  $\beta$ -linalool, menthone, pulegone, and verbenol acetate. It is concluded that the use of *R. irregularis* allows optimal growth of *S. macrostema* plants in low fertility soils and increased production of the main components of the essential oil.

**Keywords:** Essential oils, medicinal plants, *Rhizophagus irregularis*, terpenes

**Resumen:** El efecto de *Rhizophagus irregularis* sobre el rendimiento vegetal y la producción de los terpenos volátiles de *Satureja macrostema* (Benth.) Briq. (Lamiaceae), una planta medicinal nativa mexicana, fue estudiado en condiciones de invernadero. Los parámetros de crecimiento considerados en esta investigación y los componentes volátiles, fueron cuantificados mensualmente en plantas con y sin micorrizas. El aceite esencial fue colectado de la parte aérea y fue analizado por técnicas de cromatografía de gases-espectrometría de masas. La colonización de *R. irregularis* aumentó significativamente la biomasa, longitud de tallo y raíz, y la cantidad de terpenos volátiles. Los terpenos volátiles mayoritarios fueron limoneno,  $\beta$ -linalol, mentona, pulegona y acetato de verbenol. Se concluye que el uso de *R. irregularis* permitió un óptimo crecimiento de las plantas de *S. macrostema* en suelos de baja fertilidad, con un aumento de los componentes principales del aceite esencial.

**Palabras clave:** Aceites esenciales, plantas medicinales, *Rhizophagus irregularis*, terpenos

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## INTRODUCTION

Most of the medicinal plants cultivated in Mexico are not native to the country, such as chamomile (*Matricaria recutita*), lavender (*Lavandula angustifolia*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), marjoram (*Origanum majorana*) and spearmint (*Mentha spicata*), among others (Estrada *et al.*, 1995). However, there is very little documented research of agronomic factors affecting native medicinal plant's qualitative and quantitative characteristics. It is known that mineral fertilization increases biomass production, which is associated with increasing content and number of components of secondary metabolites of economic interest, such as volatile oils (Zelyazkovet *et al.*, 2009).

Furthermore, it has been reported that application of Arbuscular Mycorrhizal (AM) symbiosis contributes to the growth of the host plant and to the synthesis, accumulation, and quality of some secondary metabolites such as terpenoids, flavonoids, and phytoalexins (Akiyama & Hayashi, 2002; Larose *et al.*, 2002; Yao *et al.*, 2003). Arbuscular mycorrhizal fungi (AMF) affect secondary metabolism and the production of active compounds of medicinal plants and thus influence the quality of herbal medicines (Zeng *et al.*, 2013).

Mycorrhizal colonization increased the content and composition of essential oils produced by plants of *Ocimum basilicum*, *Mentha arvensis*, *Santolina chamaecyparissus*, *Salvia officinalis*, *Lavandula angustifolia*, *Geranium dissectum*, *Origanum dictamnus*, and *Artemisia annua* (Freiteas *et al.*, 2004; Rapparini *et al.*, 2008; Karagiannidis *et al.*, 2012).

Due to the high demand for native medicinal plants collected from the wild, investigations are needed for clarifying the factors determining the variation of volatile compounds in order to increase the yields of essential oils of plants that grown in wild and greenhouse (Estrada, 2002). Such is the case of *Satureja macrostema* (Benth.) Briq. (Lamiaceae) commonly known as "nurite" that is used in traditional medicine in central-western Mexico (Bello, 1993; Rzedowski & Rzedowski, 2001). *S. macrostema* contains a mixture of flavonoids that have been extensively studied for its antioxidant effects (Perez-Gutierrez & Gallardo-Navarro, 2010), but other volatile compounds are also reported, such as limonene, pulegone, and menthone, which are considered to be antimicrobials (Bello, 2006).

Wild nurite plants are over-collected to satisfy the demand from regional and national markets (Bello, 2006), but there are no programs designed for its domestication involving propagation systems, cultivation practices in the greenhouse, and studies of volatile oil content variation. Therefore, the aim of our investigation was to determine the effect of *Rhizophagus irregularis* MUCL 41833 on the content of volatile compounds produced by *S. macrostema* plants grown in the greenhouse.

## Material and methods

### *Rhizophagus irregularis* MUCL 41833

Single cultures of *Rhizophagus irregularis* MUCL 41833 were subcultured and propagated in carrot hairy roots on low mineral media minimal (M medium) (Balaji *et al.*, 1995). Several thousand spores were obtained in a period of 4 months, which were isolated by solubilization of the medium with citrate buffer (10 mM), and afterwards maintained in sterile deionized water (Cranenbrouck *et al.*, 2005).

### Plant material and mycorrhizal inoculation

Plants of *Satureja macrostema* (Benth.) Briq. (Lamiaceae) were propagated from seeds collected from plantations established in the experimental area of Nuevo San Juan Parangaricutiro, Michoacán, Mexico (19°25'23''N, 102°07'47''W). Seeds were sterilized for 10 min in NaClO solution (1.2%), and then rinsed three times for 5 min in sterile deionized water. Seeds were planted in germination trays containing soil:sand mix (v/v, 1:1, pH 5.5) in greenhouse conditions (16 h day length, temperature between 22-25° C, 70% relative humidity), and watered three times a week. Previously, the soil:sand mix was sterilized at 180° C for 2 h (Copetta *et al.*, 2006). After 15 days of germination, seedlings (5 cm in height) with fully expanded cotyledonary leaves and well developed roots were individually transferred to pots containing soil:sand mix sterilized (1.5 kg, v/v, 3:1, pH 5.5). At 7 days after transplantation, soil at the base of *S. macrostema* plants was gently pushed aside to expose portions of the roots system, and then the inoculation was realized with the *R. irregularis* (50 spores/plant). Roots were covered with soil immediately after inoculum application.

The *S. macrostema* plants with and without mycorrhiza were grown in greenhouse conditions and irrigated every 20 days, for up to twelve weeks, with

a low phosphate level (1 mM  $\text{KH}_2\text{PO}_4$ , modified Hoagland's solution) (Hoagland & Arnon, 1950).

### Colonization percentage

At 30 and 90 days of inoculation, the plants were taken out of the pots and washed carefully to remove substrate from roots. A simple sample (2 g) of roots of test plants was taken to determine mycorrhizal colonization, by staining with trypan blue in lactophenol (Phillips & Hayman, 1970). The percentage of colonization of the root system was quantified by the grid intersect method, which estimates the percentage of root colonized. Is a procedure whereby the presence or absence of colonization at each intersection of root and gridline is noted, after dispersing the roots above a grid of square drawn on a Petri dish and observing under a dissecting microscope at X40 magnification (McGonigle *et al.*, 1990).

### Growth parameters

Seven plants from each treatment were harvested at 30, 60 and 90 days after inoculation with *R. irregularis* for recording various morphological parameters: dry weight (dry wt) of shoots and roots (g/plant); shoot and root length (cm). Shoot and root dry weights were determined by weighing samples after being washed, dried with filter paper, and then drying for 24 h at 80° C. Plants were distributed in a randomized design and the data on morphometric parameters were compared by one-way ANOVA ( $P < 0.05$ ). Standard errors were calculated for all data.

### GC-MS analysis

Aerial parts (apical shoot with 3-4 leaves) from three specimens of plants with and without mycorrhiza were collected early in the morning. Plant material was extracted immediately by vigorous shaking with n-hexane (10 mL of n-hexane per gram of sample), the extract was macerated for 5 days (4° C), filtered (Whatman N° 1 filter paper), and n-hexane was evaporated to dryness at 45° C in vacuum evaporator. The residues were dissolved in n-hexane at 1 mg/mL and were analyzed by gas chromatography-mass spectrometry (GC-MS).

GC-MS analyses were performed on a GC-HP6890-GCMS HP5973 fused silica analytical HP-5 MS capillary column (25 m x 0.25 mm x 0.25  $\mu\text{m}$  film thickness). The temperature programmed for the gas chromatography was as follows: initial temperature of 60° C held for 5 min, linear gradient of 5° C/min to 300° C, a final hold time of 30 min.

The injector temperature was 260° C, and injection was performed in a split ratio 1/30. The carrier gas was helium (99.99% purity, 1.0 mL/min). Injection volume of each sample was 1  $\mu\text{L}$ . Retention indices for all compounds were determined using n-alkanes (C8 to C20) as standards. Compounds were identified by comparison of their MS spectra with the NIST02 mass spectral library (National Institute of Standards and Technology), as well as by comparison of their retention indices with those described by Adams (2007). Quantitative determination was based on the total ion count detected by the GC-MS. Statistical analysis of the data was carried out by analysis of variance ( $P \leq 0.05$  significance level,  $n = 3$ ).

## RESULTS AND DISCUSSION

At 30 days of *R. irregularis* inoculation *S. macrostema* plants showed a percentage of colonization of 30% with observation of hyphae and arbuscules. The mycorrhizal colonization increased proportionally at 60 and 90 days, with 60 and 80%, respectively. Such values are considered acceptable for other medicinal plants to these times of cultivation (Freiteas *et al.*, 2004; Binet *et al.*, 2011; Karagiannidis *et al.*, 2012). In *Catharanthus roseus*, mycorrhizal colonization with *G. fasciculatum* was 85% at 150 days (Karthikeyan *et al.*, 2009). These results demonstrate that *S. macrostema* is a plant with high degree of mycotrophy.

Plants of *S. macrostema* inoculated with *R. irregularis* showed improved growth and development when compared to control plants (non-mycorrhizal), being larger the root and shoot biomasses (dry weight) of inoculated plants. At 90 days, the length of shoots and roots, and the biomass was significantly higher in mycorrhizal plants (Table 1). The results are in agreement with the findings of earlier work by Gupta & Janardhanam (1991), who recorded a two-fold increase in growth and three-fold increase in biomass production as compared to plants without mycorrhiza in *Cymbopogon martini* on inoculation with *Glomus aggregatum*. A similar response was also observed in ten medicinal plants (*Abrus precatorium*, *Cynodon dactylon*, *Euphorbia tirucalli*, *Gymnema elegans*, *Hemidesmus indicus*, *Ocimum basilicum*, *Plumbago zeylanica*, *Phyllanthus amarus*, *Sida acuta* and *S. rhombifolia*) inoculated with three AMF species (*G. mosseae*, *G. fasciculatum* and *G. monosporum*), recording the highest AMF infection (86%) in *Arbus precatorius*, and the lowest in *Phyllanthus amarus* (36%) (Kumar & Muruges, 2002).

In a study by Coppeta *et al.* (2007), inoculation of sweet basil (*Ocimum basilicum* var. Genovese) plants with *Gigaspora rosea* significantly increased shoot length (54.7 cm) and number of nodes (9.0) in comparison with control plants (46.9 cm, 7.7 nodes) and the other fungal treatments (*Glomus mosseae* or *Gigaspora margarita*) at 63 days of culture. The authors reporting that mycorrhizal inoculation was advantageous in terms of obtaining healthy vigorous seedlings, and a higher biomass of plants that grew better in the field.

The reason for these effects may be the formation of external mycelium surrounding the roots by *R. irregularis*. The extramatrical hyphae produced by AMF act as extensions of roots increasing the surface area of the root system and making it more efficient for absorption of water, and for diffusion of limited nutrients. This effect is more pronounced in phosphorus deficient soils (Bagyaraj & Reddy, 2000). It has been demonstrated that plants inoculated with AMF utilize more soluble phosphate from soil than non-mycorrhizal plants (Antunes & Cardoso, 1991).

Table 1

**Effect of *R. irregularis* inoculation on the growth of *Satureja macrostema* plants in greenhouse**

Growth parameters	Non-mycorrhizal plants			Mycorrhizal plants		
	30d	60d	90d	30d	60d	90d
Root dry wt (g/plant)	2.7	4.9	7.2	5.9	7.6	12*
Shoot dry wt (g/plant)	5.8	8.8	11.2	9.2	18.4	24.6*
Root length (cm)	8.82	17	24.2	14.2	20.8	28.1*
Shoot length (cm)	18.2	29.7	34.6	22.1	42.1	48.2*

\*Significant difference (n=7, P &lt; 0.05)

The volatile compounds were identified and quantified during the 90 days of experimentation, the major terpenes found were limonene,  $\beta$ -linalool, menthone, pulegone, and verbenol acetate. However, the content of these terpenes varied according to plant development and mycorrhizal colonization. Table 2 summarizes the main volatile compounds identified by GC-MS in aerial parts of control plants of *S. macrostema* after 30 days under greenhouse conditions. Pulegone (58.3%) and  $\beta$ -linalool (30.4%) were the main compounds, followed by limonene (1.5%), menthone (2.8%) and verbenol acetate (7.0%).

The volatile terpenes content did not change

significantly during culture of *S. macrostema* non-mycorrhizal plants, with the exception of pulegone that dramatically increased at 60 days of culture (33.4%), diminishing again at 90 days (Table 3). However, volatile terpenes showed a tendency to increase in mycorrhizal plants, in which they were produced at larger amounts in non-mycorrhizal plants beginning at 30 days of culture. At 90 days, the production of limonene,  $\beta$ -linalool, menthone and verbenol acetate was highest in plants with mycorrhizal in comparison with non-mycorrhizal plants. However, pulegone content was most abundant at 60 days, diminishing at 90 days (Table 3).

Table 2

**Volatile compounds observed in aerial parts of *Satureja macrostema* at 30 days under greenhouse conditions.**

Compound <sup>a</sup>	Rt <sup>b</sup> (min)	IKI <sup>c</sup>	GC area <sup>d</sup> (%)
Limonene	9.08	1024	1.5
$\beta$ -Linalool	11.14	1095	30.4
Menthone	13.05	1164	2.8
Pulegone	15.15	1233	58.3
Verbenol acetate	17.87	1340	7.0

<sup>a</sup>Identified by GC-MS; <sup>b</sup>Rt: retention time; <sup>c</sup>Experimental Kovat's Retention Index; <sup>d</sup>Quantified by GC.

**Table 3**  
**Content of volatile compounds from the aerial part of *Satureja macrostema* non-mycorrhizal and mycorrhizal plants.**

	Content ( $\mu\text{g/g}$ fresh wt $\pm$ Sd)					
	Non-mycorrhizal plants			Mycorrhizal plants		
	30d	60d	90d	30d	60d	90d
Limonene	1.05 $\pm$ 0.07	1.07 $\pm$ 1.13	1.21 $\pm$ 0.19	2.13 $\pm$ 0.11	2.19 $\pm$ 0.19	4.49 $\pm$ 0.37*
$\beta$ - Linalool	5.07 $\pm$ 0.41	6.32 $\pm$ 0.57	6.7 $\pm$ 0.33	6.45 $\pm$ 0.71	8.4 $\pm$ 0.66	12.22 $\pm$ 1.13*
Menthone	0.667 $\pm$ 0.09	0.991 $\pm$ 0.11	1.17 $\pm$ 0.18	1.03 $\pm$ 0.97	1.47 $\pm$ 0.13	2.97 $\pm$ 0.21*
Pulegone	11.5 $\pm$ 1.18	16.71 $\pm$ 1.81	12.0 $\pm$ 1.31	18.1 $\pm$ 1.19	34.4 $\pm$ 2.99*	22.7 $\pm$ 2.19
Verbenol acetate	1.11 $\pm$ 0.09	1.14 $\pm$ 0.14	1.16 $\pm$ 0.17	1.16 $\pm$ 0.18	1.28 $\pm$ 0.13	2.42 $\pm$ 0.27*

Sd: Standard deviation; \*Significant difference (n=3, P  $\leq$  0.05)

The content in mycorrhizal plants of  $\beta$ -linalool, menthone, pulegone, and verbenol acetate increased almost 100% respect to non-mycorrhizal plants at 90 days, but limonene incremented to 371%. The results of this experiment show that the application of *R. irregularis* on the roots of the plants of *S. macrostema* induces an increment of volatile terpenes content with respect to non-inoculated counterparts. The significant increase of the concentration of volatile terpenes has also been observed in Apiaceae species (*Anethum graveolens*, *Coriandrum sativum* and *Foeniculum vulgare*) inoculated with *Glomus* spp (Kapoor *et al.*, 2002; Kapoor *et al.*, 2004); and in Lamiaceae plants (*Mentha arvensis*, *Ocimum basilicum* and *Origanum vulgare* ssp. *Hirtum*) (Gupta *et al.*, 2002; Copetta *et al.*, 2006; Toussaint *et al.*, 2007; Morone-Fortunato & Avato, 2008).

The decrease in the content of pulegone at 90 days is related with the increase of limonene (Table 3). This behavior has been reported in plants of *Mentha piperita* where pulegone content is highly influenced by the stage of development of the plant and by the environmental conditions (Mahmoud & Croteau, 2003).

Essential oils of different plants exert activity antioxidant and anti-inflammatory, these effects are attributed to terpene components (Cardona & Mejia, 2009; Daniel *et al.*, 2009). The antimicrobial, antioxidant or anti-inflammatory activity of essential oils of some species of *Satureja* have been demonstrated *in vivo* or *in vitro* models (Amanlou *et al.*, 2005; Ozkan *et al.*, 2007; Hajhashemi *et al.*, 2011). The high limonene content in mycorrhizal *S. macrostema* plants is very important by the properties attributed, it is used in pharmaceutical industry as a

diuretic, stimulant and a carminative (Bailer *et al.*, 2001; Singh *et al.*, 2005; Callan *et al.*, 2007). These results shown that mycorrhizal *S. macrostema* plants produced in a greenhouse could be an alternative for production of plants with high contents of volatile compounds.

## CONCLUSIONS

The mycorrhizal colonization by *Rhizophagus irregularis* in *Satureja macrostema* had a positive effect on plant performance and increased contents of major volatile compounds. In all cases, pulegone was the main volatile component, and limonene production was highest at 90 days of cultivation.

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